**Advances in Brief**

**p14ARF Promoter Hypermethylation in Plasma DNA as an Indicator of Disease Recurrence in Bladder Cancer Patients**

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**Abstract**

**Purpose:** Several genes are reported to be implicated in bladder carcinogenesis, including p53, p16INK4a, pRb, erbB-2, Cyclin D1, H-ras, EGF R, and c-myc. Gene alterations in plasma DNA identical to those observed within the tumor have been detected in various types of neoplasia.

**Experimental Design:** We analyzed loss of heterozygosity in six microsatellite markers (D17S695, D17S654, D13S310, TH2, D9S747, and D9S161), p53 and K-ras mutations, and the promoter status of p14ARF and p16INK4a in the mononuclear normal blood cells, tumor, and plasma DNA of 27 bladder cancer patients. We also studied the distribution of several clinicopathological parameters in these patients in regard to molecular alterations.

**Results:** Seventeen (63%) cases displayed the same alteration in plasma and tumor DNA (some patients showed more than one alteration simultaneously). Plasma p14ARF promoter hypermethylation was associated with the presence of multicentric foci (P = 0.03), larger tumors (P = 0.01), and relapse of the disease (P = 0.03). Plasma loss of heterozygosity was also linked to disease recurrence (P = 0.02).

**Conclusions:** The results indicate that p14ARF aberrant promoter methylation could be involved in bladder carcinogenesis and that plasma DNA is a potential prognostic marker in urinary bladder cancer.

**Introduction**

Urinary bladder cancer is the fifth most common tumor in Western societies. Its occurrence increases with age and it is greater in men (with a sex ratio between 3:1 and 4:1; Ref. 1). The risk of bladder cancer is doubled in smokers (2), but there are other risk factors (3). About 95% of bladder neoplasms are transitional cell carcinomas. The remainder are squamous tumors, adenocarcinomas, and other subtypes. At the time of diagnosis, >60% of the transitional carcinomas are papillary noninvasive (Ta), 10–20% show invasion limited to the lamina propria (T1), and 20% present muscle or deeper infiltration (T2-T4; Refs. 4, 5). Between 15 and 30% of bladder tumors show grade and stage progression (3, 6). Despite radical local therapy, half the patients with muscle-invasive bladder tumors die from metastatic disease (7). Bladder cancer is the result of monoclonal genetic changes, and the multiple synchronous or metachronous tumors are derived from micrometastatic foci that have migrated from the original site rather than from a polyclonal mutation (8–10).

Many studies have sought predictive markers in bladder cancer patients. Several genes are involved, including erbB-2, EGF R, c-myc, Cyclin D1, H-ras, p53, p16INK4a, and pRb. Frequent deletions at 2q, 3p, 4q, 5q, 6q, 7p, 9q, 11p, 11q, and 13q, and frequent gains of 1q, 3q, 5p, 6p, chromosome 7, 8q, 17q, and 20q may pinpoint the location of other, yet unidentified tumor suppressor genes and oncogenes with a role in bladder cancer (11–15).

Free-circulating tumor DNA has been detected in peripheral blood of both healthy subjects and tumor patients. The mean concentration of soluble DNA in plasma was 14 ng/ml in control subjects (16) and 180 ng/ml in cancer patients (17). Gene alterations identical to those observed within the tumor have been detected by PCR in the plasma DNA of patients with various types of tumor (18–29). The prognostic implications of circulating plasma DNA in patients with malignancies regarding the outcome of the disease are being examined. A correlation with poor survival in pancreatic carcinomas has been reported (30), and two studies about the follow-up of lung cancer and melanoma patients have been published recently (31, 32). A novel plasma DNA utility has been used to screen somatic mutations that are frequent in tumors and detect them in a preclinical phase of the disease (33).

The presence of microsatellite alterations in plasma DNA of bladder cancer patients has been reported recently (34, 35). The present study aims to: (a) detect tumor DNA at diagnosis in the plasma of such patients; and (b) analyze the distribution of various clinicopathological parameters. We characterized tumor DNA in plasma by checking alterations in microsatellite markers at described deleted loci, in tumor suppressor genes, and in the K-ras oncogene. To our knowledge, this is the first analysis of p14ARF promoter status in bladder cancer.

**Materials and Methods**

**Tissue Sampling and DNA Extraction.** Between April 1999 and June 2001, 27 samples from tumor tissue were ob-
tained immediately after TUR\(^3\) from patients with a diagnosis of bladder cancer (26 transitional cell carcinomas and 1 squamous tumor). They were then snap-frozen in liquid nitrogen and stored until processing. All of the specimens underwent histological examination (a) to confirm the diagnosis; (b) to confirm the presence of tumor and evaluate the percentage of tumor cells in each sample; and (c) to perform pathological staging. All of the specimens were examined for the presence of tumor and evaluated the percentage of tumor cells.

Bladder cancer (26 transitional cell carcinomas and 1 squamous cell carcinoma) was classified by the Tumor-Node-Metastasis system, where T is the presence or absence of the primary tumor. DNA was extracted from tumor tissues, normal blood cells, and plasma immediately. DNA was extracted from tumor tissue samples and peripheral blood mononuclear cells by a non organic method (5–4520 kit; Oncor, Gaithersburg, MD). Plasma DNA was purified on Qiagen columns (Quiamp Blood kit; Qiagen, Hilden, Germany) following the protocol for blood and body fluids, modified as described elsewhere (26).

**Analysis of Clinicopathological Parameters.** The following information was obtained from the medical records of the 27 patients: (a) birth and diagnosis dates; (b) radiological lymph node enlargement; (c) presence of multicentric disease; (d) tumor size; (e) histological subtype; (f) histological grade; (g) invasiveness; (h) pathological stage; (i) treatment; and (j) recurrences. All of the tumors were graded according to the number of mitotic elements and atypias. Pathological stage was classified by the Tumor-Node-Metastasis system, where T is the presence of the tumor, N the lymph nodes affected, and M the metastases to other organs. All of the patients underwent TUR, and after staging, 9 patients were followed without any adjuvant treatment, 15 received intravesical therapy based on bacillus Calmette-Guérin, and 3 underwent radical cystectomy for invasion or progression of bladder cancer.

**Microsatellite Analysis and PCR Conditions.** PCR was performed in a 10-μl reaction volume containing 50 ng/μl of sample DNA (tumor, mononuclear blood cells, or plasma DNA), 0.2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 1 μl of 10 × PCR buffer [100 μM Tris-HCl (pH 8.3), 500 mM KCl], 200 μM dNTP, 0.6 μM of each primer, and a range of concentrations of KCI and MgCl\(_2\) depending on the polymorphic marker. A 30-cycle amplification was carried out in a thermal cycler (Perkin-Elmer, Cetus, Foster City, CA). Six microsatellite markers were used to determine LOH on the following chromosomes: (a) 17: D17S695 (36) and D17S654 (37); (b) 13: D13S310 (38); (c) 11: TH2 (39); and (d) 9: D9S747 (accession no. GDB 335542) and D9S161 (40). Annealing was at 63°C, 65°C, 56°C, 58°C, 63°C, and 60°C, respectively. The allelic bands were separated by mixing the 10 μl of the PCR products with 10 μl of loading buffer (0.02% xylene cyanol and 0.02% bromphenol blue). Electrophoresis was run on nondenaturing 8% polyacrylamide gels for 3–5 h at 500 V at room temperature. The allelic band intensity was then detected by silver staining and analyzed by densitometry. The gel image was captured using a GS-690 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA), digitalized in 400 dpi, and analyzed using Multi-Analyst/PC (Bio-Rad Laboratories). LOH was accepted when the signal of the allele was >75% lower than that observed in the normal counterpart DNA, considering that variations in the amplification process and especially the presence of small amounts of normal cells in the tumor tissue or, in our study, of normal blood cell DNA in plasma DNA, could lead to the expression of various band intensities in the total absence of one allele.

**Mutational Study of the p53 Gene.** To detect point mutations in the conserved exons of p53, PCR-SSCP was performed as described elsewhere (26). PCR was carried out in a final volume of 10 μl containing 50 ng of DNA template, 1 μl of 10 × PCR buffer, 0.2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc.), 200 μM dNTP, 0.6 μM of each primer, and a range of concentrations of KCl and MgCl\(_2\) depending on the exon amplified. For PCR amplifications, the samples underwent 30 cycles. The amplified products were denatured by adding 10 μl of denaturing stop solution, which contained 98% formamide, 10 ml/liter ethylenediamine (pH 8.0), 0.02% xylene cyanol, and 0.02% bromphenol blue, heated to 95°C for 5 min and rapidly cooled on ice. Electrophoresis was run on nondenaturing 10% polyacrylamide gels for 12–15 h at 250 v. The allelic band intensity on the gels was detected as described above. The specimens that showed a differential band at SSCP were amplified to obtain templates for DNA sequencing. These amplifications were independent of those used for SSCP analysis. Amplified DNA fragments were purified from 0.9% agarose gels using the Geneclean kit (Bio-101 Inc., La Jolla, CA) and sequenced using an ABI Prim 377 DNA apparatus (PE Applied Biosystems).

**Mutational Study of the K-ras Oncogene.** PCR amplification of the K-ras gene, using 50 ng of DNA as template, was carried out in a 10-μl reaction volume with a final concentration of 1 × PCR buffer and 0.2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc.), 200 μM dNTP mix, 0.6 of each primer, 2.5 mM MgCl\(_2\), and distilled H\(_2\)O to reach the total volumes. Each sample was denatured at 94°C for 5 min and subjected to 30 cycles (94°C for 30 s, 58°C for 40 s, and 72°C for 30 s), followed by a final 7-min extension at 72°C. The amplified products were mixed with 10 μl of denaturing stop solution (see “Mutational study of the p53 gene”), heated to 95°C for 5 min, and rapidly cooled on ice. The samples were electrophoresed on nondenaturing 12% polyacrylamide gels at 250 v overnight at 4°C. Products were visualized by nonradioisotopic means. The primers used for amplification of exon 1 of K-ras, which contains codons 12 and 13, were: 5’GACTGAAATAAACCTTGTGTAGT and 5’TATTTGTTTGATCATATCGTCC. All of the specimens showing a differential band at SSCP were sequenced following the method used for the p53 gene.

**Promoter Hypermethylation.** p16INK4a and p14ARF aberrant promoter methylation was analyzed in tumor, mononuclear blood cells, and plasma DNA. DNA methylation patterns in the CpG island of the p16INK4a and p14ARF genes were determined by methylation-specific PCR as described elsewhere (41). Briefly, 1 μg of DNA from tumor, blood cells, and plasma was denatured by NaOH, and subsequently treated with

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\(^3\) The abbreviations used are: TUR, transurethral resection; dNTP, deoxyribonucleotide triphosphate; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.
hydroquinone and sodium bisulfite at 53°C for 16 h. Modified DNA samples were purified using the Wizard DNA purification resin following the manufacturer instructions (Promega) and eluted into 50 μl of water. NaOH was added to complete the modification followed by ethanol precipitation. Resuspended DNA was used in a PCR reaction. Primer sequences and PCR conditions for p16INK4a and p14ARF are reported elsewhere (41, 42). Placental DNA treated with Sss1 methyltransferase and DNA of Raji, KMH2, and L-540 cell lines were used as positive controls for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated genes. Samples were electrophoresed on nondenaturing 8% gels at 450 v for 2–3 h and visualized by nonradioisotopic techniques.

**Statistical Analysis.** The variables were contrasted using the χ² test. Differences are considered significant where P < 0.05. Statistical analyses were performed using the EPI-INFO package, version 6.04.

### Results

**Microsatellite Analysis.** Fifteen bladder carcinoma patients (56%) showed allelic loss in at least one locus. Nine of these (60%) had the same microsatellite alteration in their plasma DNA. The marker D9S161 showed the highest individual rate of LOH in tumor DNA (30%), but D9S747 showed the highest correlation between tumor and plasma DNA (100%; Fig. 1A). In 6 cases, plasma DNA revealed an additional alteration, which was not present in tumor DNA (Fig. 1B). Although 3 of these cases showed LOH in their tumor and plasma samples for some of the other microsatellite markers.

**p53 Gene Mutations.** Only 1 tumor patient showed a mutation in exon 8 or p53, which was not found in the corresponding plasma sample (Fig. 1C). Another patient presented a nucleotide change in exon 6 of p53 in both normal blood cells and tumor DNA, but again, it was not detected in plasma. The incidence of p53 mutations was lower (3.7%) than in other studies (43, 44), but in a recent report, 1% of p53 mutations were detected by microarray technology (45).

**K-ras Gene Mutations.** In 2 cases, the same K-ras mutations were identified in both tumor tissue and plasma (Fig. 1D). One case showed de novo K-ras mutation in plasma, which was not detected in its tumor counterpart. This case showed LOH for D9S747 and D13S310 microsatellite markers in the tumor and plasma samples. Mutations in the K-ras oncogene are rare in human bladder cancers (46).

**Methylation Status of the p14ARF and p16INK4a Promoters.** Methylation of 5′ regulatory regions of CG dinucleotides, called CpG islands, is a well-established mechanism of transcriptional repression (47). p14ARF and p16INK4a are candidates for hypermethylation-associated inactivation, because they contain documented CpG islands that can be silenced by this epigenetic alteration in many tumor types (48–50). Of the bladder tumor samples amplified, 15 (56%) and 5 (18%) presented p14ARF and p16INK4a promoter hypermethylation, respectively. Of these, 13 (87%) and 2 (40%) showed p14ARF and p16INK4a promoter hypermethylation in plasma samples, respectively (Fig. 1, E and F). All of the cases with p16INK4a promoter hypermethylation were positive for p14ARF promoter hypermethylation. Bisulfite-modified DNA from the 27 normal blood cells samples was not amplified with either primers. To our knowledge, p14ARF promoter hypermethylation has not been analyzed in bladder cancer patients and we cannot compare our results. However, the percentage found for p16INK4a promoter hypermethylation is within reported ranges for bladder carcinomas (51, 52).

**Correlations between Clinicopathological Parameters and Molecular Changes.** Of the 27 patients, 21 (78%) showed in the tumor sample at least one of the alterations analyzed. A significant correlation was found between tumor p14ARF promoter hypermethylation and multicentric foci (P = 0.04), tumor size (P = 0.006), bladder muscle invasion (P = 0.01), and stage (P = 0.01). Nearly statistically significant was lymph nodes enlargement (P = 0.05; Table 1). No statistical associations were found in regard to LOH in the tumor samples.

Twenty cases (74%) showed at least one of the alterations in plasma DNA (17 cases displayed the same alterations in their tumor and plasma samples, and 3 cases showed LOH in plasma but not in their tumor samples for any of the microsatellites analyzed). There was a significant association between p14ARF hypermethylation in plasma DNA and multicentric foci (P = 0.03), larger tumors (P = 0.01), and relapse (P = 0.03). Close to statistical significance (P = 0.05) were lymph node enlargement (the only 3 patients harboring positive nodes showed promoter hypermethylation), invasion of the bladder muscle, more advanced stages, and the treatment applied; the 3 patients...
subjected to more aggressive therapy, *i.e.*, cystectomy, showed
p14ARF promoter hypermethylation in their plasma DNA (Table 2).
A statistical association between plasma LOH in at least one of the markers analyzed and recurrence of the disease was
detected (*P* = 0.02; Table 2).

The association with the clinical parameters was not ana-
alyzed for p53 and K-ras mutations, and p16INK4a aberrant
methylation because of the very low number of cases showing
these alterations, which could lead to misinterpretation results.

### Discussion

This is the first study to identify a possible indicator of
disease recurrence for bladder cancer in plasma DNA.

Twenty-six transitional cell carcinomas and 1 squamous
tumor, and their corresponding mononuclear blood cells and plasma
samples were analyzed for DNA microsatellite alterations, p53 and
K-ras mutations, and p14ARF/p16INK4a promoter hypermethyla-
tion. Seventeen cases showed the same alteration in plasma and
tumor DNA, indicating that this plasma DNA derives from the
primary tumor. Six cases presented a new alteration in plasma,
which was not found in the tumor counterpart. Most studies on
plasma DNA reveal a few alterations in plasma only, mainly when
microsatellite polymorphic markers are used to characterize tumor
and plasma DNA (22, 26). Some authors suggest that plasma
alterations could be artifacts (27, 28), although they are not detected
in healthy controls (26, 35). Other authors point to a heterogeneous
clonal origin (22, 24). The common multiple foci found in bladder
cancer patients may have a monoclonal origin, which is followed
by cellular spreading (8–10). Once the microfoci have migrated,
they may evolve and acquire new alterations. In this case, the
specific alterations in plasma DNA but not in the tumor sample
could be explained by the fact that this DNA proceeds from other
tumor foci which harbor the alteration, as we have described
recently for colon carcinomas (53).

<table>
<thead>
<tr>
<th>Tumor p14ARF methyl</th>
<th>Tumor p14ARF unmethyl</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>No</td>
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<tr>
<td>Tumor size</td>
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<td>&lt;3</td>
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</tr>
<tr>
<td>&gt;3</td>
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<td>Negative</td>
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</table>

* * n, number of cases; LNE, lymph node enlargement.*

### Table 2 Methylation status of tumor plasma DNA

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<th>Plasma p14ARF methyl</th>
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<td>Multicentric foci</td>
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<tr>
<td>Tumor Size</td>
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<td>9</td>
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<tr>
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<td>4</td>
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<tr>
<td>LNE</td>
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<table>
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* * n, number of cases; LNE, lymph node enlargement; IT, intraves-
ural therapy.*

The low proportion of p53 and K-ras mutations, and
p16INK4a hypermethylation in our series of bladder cancer
samples indicates that they are not suitable markers. Neverthe-
less, microsatellite alterations or p14ARF promoter hypermethyla-
tion was detected in 56% of tumors, but concordance be-
tween tumor and plasma alterations was higher for p14ARF
status (87% versus 60%). p14ARF promoter hypermethylation
has been reported in other tumors, such as colorectal (42),
gastric (54), breast (55), ovarian (56), oral squamous cell carci-
nomas (57), oligodendrogliomas (58), glioblastomas (59), and
central nervous system lymphomas (60).

It is remarkable that recurrence was associated with p14ARF
promoter hypermethylation or LOH only when the alteration was
present in plasma. This observation could be because of the spec-
ificity to find alterations in the plasma sample. Because of the
multicentric foci in bladder cancer, we could have a tumoral sample
from a focus not showing the alteration observed in the plasma.
Thus, the case would be classified inaccurately as without alter-
ation, which could modify the results.
Although alterations in plasma, LOH, and p14ARF promoter aberrant methylation could predict relapse, p14ARF promoter hypermethylation is a better prognosis marker for several reasons. Firstly, although in our subset of bladder cancer patients the proportion of these alterations in the tumors was identical (56%), concordance between presence in tumor and plasma DNA was higher for p14ARF aberrant promoter methylation. Secondly, for microsatellite analysis, some of these markers should be tested to obtain an acceptable rate of LOH. To check the promoter status of a gene, only one reaction is required.

Improvements in molecular and genetic approaches have led to the identification of tumor-derived nucleic acids in the plasma of cancer patients (20–22, 24, 26, 27, 31, 32, 37, 61, 62), although the mechanisms by which these nucleic acids are released into plasma remain unknown (62–65). It is suggested that the alterations found in the plasma DNA of these patients could be used as a prognostic factor. We have detected plasma DNA with tumor features in a series of 27 bladder cancer patients. p14ARF promoter hypermethylation may be a useful prognostic factor, because it could be associated with recurrence disease and could be clinically relevant as a noninvasive tool for the detection of relapses during the follow-up of patients.

Acknowledgments
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References
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